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Bone morphogenetic protein 15 may promote follicle selection in the hen

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ABSTRACT

In the hen, optimal ovulation rate depends on selection of a single follicle into the pre-ovulatory hierarchy. Follicle selection is associated with increased oocyte growth and changes in gene expression in granulosa cells surrounding the oocyte, in preparation for ovulation. This study investigated the expression, function and regulation of bone morphogenetic protein-15 (BMP15) during follicle development in the hen. *BMP15* mRNA expression was analyzed in the ooplasm and granulosa cells of 3 mm follicles and was confirmed to be primarily in the ooplasm. BMP15 was detected by immunoblotting in 6 and 8 mm follicles near the time of follicle selection. Expression of mRNA for BMP15 receptors (*BMPR1B* and *BMPR2*) in granulosa cells increased with follicle size, indicating that BMP15 may play an important role around follicle selection. The function of BMP15 was examined by culturing granulosa cells from 3–5 mm and 6–8 mm follicles with recombinant human BMP15 (rhBMP15). BMP15 increased expression of follicle stimulating hormone receptor (*FSHR*) mRNA and decreased anti-Müllerian hormone (*AMH*) mRNA and occludin (*OCLN*), factors associated with follicle maturation and growth in the hen. Hormonal regulation of BMP15 was assessed by whole follicle culture with estradiol (E2) which increased *BMP15* mRNA expression. The distinct expression pattern of BMP15 and its receptors, coupled with the effects of BMP15 to increase *FSHR* mRNA and decrease *AMH* mRNA and *OCLN* mRNA and protein expression suggest that the oocyte may have a role in follicle selection in the chicken.

1. Introduction

A variety of endocrine, paracrine and autocrine signals in the ovary result in growth, maturation and subsequent ovulation of one or more oocytes. Bone morphogenetic protein-15 (BMP15), also termed growth differentiation factor-9B (GDF-9B), along with growth differentiation factor 9 (GDF9), are oocyte-derived members of the transforming growth factor beta superfamily (TGFβ) that contribute to the network of signals within the mammalian follicle (Shimasaki et al., 2004). In the oviparous hen, there is little known about the early stages of follicle maturation prior to selection. Comparative biology would suggest that oocyte factors, including BMP15, could play a vital role in follicle maturation. In oviparous species, oocyte competence is especially important as proper accumulation of yolk by the oocyte is closely coordinated with steroidogenesis and follicle maturation. The aim of this study was to determine the expression, function and regulation of BMP15 in the hen ovary around the time of follicle selection.

In the hen, pre-ovulatory follicles are arranged and maintained in a size hierarchy (F1-F5), where the largest (F1) follicle will ovulate next and the second largest (F2) will follow on the subsequent day. Daily selection of a single growing follicle from a larger pool of follicles is required to maintain the pre-ovulatory hierarchy. Similar to mammals, there is increasing evidence for the involvement of TGFβ superfamily members in initiating follicle selection (Johnson et al., 2008; Kim et al., 2013; Ocón-Grove et al., 2012). Our lab has identified the oocyte as the primary source of GDF9 in the hen ovary (Johnson et al., 2005). Messenger RNA for *BMP15* has been localized by *in situ* hybridization to the hen oocyte in pre-hierarchical follicles (50 μm to 6 mm) of mature ovaries and in small follicles (50 μm to 100 μm) of immature ovaries (Elis et al., 2007). *BMP15* mRNA (by quantitative RT-PCR) in larger follicles was more abundant in the germinal disc region than in isolated granulosa cells (Elis et al., 2007).

In mammals, BMP15 plays an important role in the differentiation of somatic cells surrounding the oocyte and affects oocyte development (Gilchrist et al., 2008). There is also evidence for species-specific differences in BMP15 function among mammals

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(Al-Musawi et al., 2013; Yoshino et al., 2006). Mice null for BMP15 are sub-fertile, with little change in folliculogenesis although they have defects in ovulation (Yan et al., 2001). The Inverdale ewe, with two inactive copies of the BMP15 gene, is sterile while a single inactive copy of BMP15 in ewes results in increased ovulation rate and a heightened incidence of twins and triplets (Davis et al., 1992, 1991; Montgomery et al., 2001). BMP15 may be synthesized as a monomer and is able to form noncovalent homodimers or heterodimers with GDF9 (Chang et al., 2002; Liao et al., 2003a). Function of BMP15 is mediated through binding to serine threonine kinase BMP receptor complexes made of a type 1 and type 2 receptor: bone morphogenetic protein receptor, type IB (BMPR1B) and bone morphogenetic protein receptor, type II (BMPR2) (Moore et al., 2003; Shimasaki et al., 2004). The BMP15 receptor complex subsequently phosphorylates Smad (mothers against decapentaplegic homolog) proteins, activating the Smad1/5/8 pathway (Moore et al., 2003). Proper function of BMP15 receptors also seems important for maintaining species-specific ovulation rate. Mutations in the BMP15 receptor BMPR1B (Alk6) cause increased litter size in sheep (Fabre et al., 2003). The presence of BMP15 and GDF9 receptors has been shown in pre-ovulatory follicles of the hen and mRNA expression was characterized in the granulosa and theca layers of small, 6–8 mm follicles (Al-Musawi et al., 2007; Onagbesan et al., 2003). There are no data, however, on mRNA expression levels of the receptors from smaller follicles.

In the hen, there are distinct developmental changes in granulosa cell mRNA expression of follicle stimulating hormone receptor (FSHR), anti-Müllerian hormone (AMH) and occludin (OCLN), which play important roles in follicle selection. One follicle in the pool of 6–8 mm follicles has a higher abundance of mRNA for *FSHR* which may allow the granulosa cells to become FSH-responsive, resulting in increased cAMP production and expression of genes associated with granulosa cell differentiation (Woods and Johnson, 2005). *AMH* mRNA expression in the hen significantly declines as follicle size increases; it is most abundant in small follicles approximately 1 mm in diameter (Johnson et al., 2008). mRNA expression for *AMH* significantly decreases from 5 mm follicles to 6–12 mm follicles (Johnson et al., 2008), when follicle selection is believed to occur. Although the function of AMH in the hen is not known, the temporal expression of *AMH* in granulosa cells parallels mammalian expression during follicle development with higher mRNA expression in preantral-stage follicles (Baarends et al., 1995). AMH null mice are fertile but develop an increased number of small growing follicles, ultimately resulting in a significant premature depletion of follicles from the ovary (Durlinger et al., 2002). The regulated temporal expression of AMH in the hen could allow orderly selection of a single follicle into the pre-ovulatory hierarchy.

Yolk production and accumulation is imperative for follicle growth. Estradiol (E2) produced in small follicles of the hen stimulates the production of yolk in the liver and the yolk is subsequently incorporated into the oocyte by receptor-mediated endocytosis. Chickens have a unique very low density lipoprotein receptor homolog (LR8; LDL receptor relative with eight binding repeats) localized on the oocyte membrane (Shen et al., 1993). Although small follicles express LR8 mRNA and protein, they fail to take up yolk. Tight junction proteins including occludin (OCLN) regulate paracellular permeability (Anderson and Van Itallie, 1995; Furuse et al., 1993). It has been suggested that OCLN, present in granulosa cells, may regulate access of yolk to the oocyte surface around the time of follicle selection (Schuster et al., 2004). Expression of protein for OCLN is lower in the granulosa cells of hierarchical follicles actively accumulating yolk as compared to small follicles (Schuster et al., 2004).

To date, the function of BMP15 in the hen has been examined only in later stages of follicle development after selection (Elis et al., 2007). We hypothesize that BMP15 may be important in regulating aspects of early follicle selection in the hen ovary because of its role in follicle development in mammals and the identification of *BMP15* mRNA in the hen ovary.

2. Materials and methods

2.1. Animals

Single-comb White Leghorn hens (Babcock B300 strain) were used in all the experiments in this study. Hens were housed individually in laying cages and maintained on a lighting schedule of 15 h of light and 9 h of darkness. All hens received *ad libitum* access to feed and water. Egg production was recorded daily. Hens with consistent laying patterns during their first year of lay were selected, euthanized and their ovaries collected within 2 h of ovulation and immediately placed in ice-cold Krebs-Ringer bicarbonate buffer. The Institutional Animal Care and Use Committee of Cornell University approved all animal procedures and techniques.

2.2. Tissue isolation and quantitative real-time PCR

Ooplasm and granulosa cells from 3 mm follicles were collected from individual hens (n = 4) according to the procedure described by Wang et al. (2007). In a separate experiment, granulosa cell layers from 3–5, 6–8, 9–16 mm follicles and whole <2 mm follicles were isolated (n = 4–6 hens). RNA was extracted using RNeasy Mini Kit with an on-column ribonuclease-free deoxyribonuclease treatment (Qiagen Inc., Valencia CA). Reverse transcriptase reactions were performed using 1 μg of total mRNA in a 20 μl volume using the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA). Primer pairs (*BMPR1B*, *BMPR2*, *BMP15*, *OCLN*) spanning introns were designed using Primer3 software for SYBR green assays (Rozen and Steve Skaletsky, 1998). For *FSHR* and *AMH*, TaqMan primers and probes were designed with Primer Express Software 2.0 (Applied Biosystems, Foster City, CA). Primer and probe sequences can be found in Table 1. Primer efficiencies, determined by the slope of the standard curve, were within 90.4–103.2% for all primers used.

Quantitative real time PCR reactions (AB StepOnePlus Real-Time PCR System) were set up in a volume of 25 μl in duplicate with a final concentration of 1X Power SYBR Green (Applied Biosystems, Foster City, CA, USA) and 300 nM of primer pairs. All samples were run in duplicate and reactions were normalized to *18S* rRNA (Applied Biosystems, Foster City, CA, USA). *18S* has been used as a reference RNA in the ovary (Haugen and Johnson, 2010; Ocón-Grove et al., 2012) and in our experiments, *18S* expression was not significantly different by tissue or treatment. Control reactions containing no template and reactions lacking reverse transcriptase were also run. PCR reactions for the TaqMan assays (*FSHR*, *AMH)* were conducted in a 25 μl reaction in duplicate with 1x TaqMan universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 900 nM of primers and 250 nM of probe or 50 nM of *18S* primers and 200 nM of probe (Applied Biosystems, Foster City, CA)

2.3. Western blot

Protein lysates were made using Tris-Triton lysis buffer from 2, 6 and 8 mm whole follicles and granulosa cells from 6–8 mm follicles were used from different hens (n = 3 individual hens). Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific Waltham, MA). Forty micrograms of total protein lysate were subjected to SDS-PAGE under denaturing conditions on a 12% Tris-HEPES gel (Pierce Biotechnology Rockford, IL).